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Early susceptibility for epileptiform activity in malformed cortex

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Abstract

Despite early disruption of developmental processes, hyperexcitability is often delayed after the induction of cortical malformations. In the freeze-lesion model of microgyria, interictal activity cannot be evoked in vitro until postnatal day (P)12, despite the increased excitatory afferent input to the epileptogenic region by P10. In order to determine the most critical time period for assessment of epileptogenic mechanisms, here we have used low-Mg²⁺ aCSF as a second hit after the neonatal freeze lesion to examine whether there is an increased *susceptibility* prior to the overt expression of epileptiform activity. This two-hit model produced increased interictal activity in freeze-lesioned relative to control cortex. We quantified this with measures of incidence by sweep, time to first epileptiform event, and magnitude of late activity. The increase was present even in the P7-9 survival group, before increased excitatory afferents invade, as well as in the P10-11 and P12-15 groups. In our young adult group (P28-36), the amount of interictal activity did not differ, but only the lesioned cortices produced ictal activity. We conclude that epileptogenic processes begin early and continue beyond the expression of interictal activity, with different time courses for susceptibility for interictal and ictal activity.

Keywords

Developmental Epilepsy; low magnesium aCSF; two-hit epilepsy; Microgyria; Neocortex

Introduction

Cortical malformations, induced by errors in developmental steps or loss of neurons during the formation of the cortical plate, necessarily affect the subsequent course of maturation (Squier and Jansen, 2010). Interestingly, despite this early disruption of cellular processes and connections, hyperexcitability is often delayed in both experimental epilepsy models (Cobos et al., 2005; Jacobs et al., 1999a; Trotter et al., 2006; Zeng et al., 2011) and the clinical population (Bartolomei et al., 1999; Kobayashi et al., 2001; Park et al., 2006; Rademacher et al., 2000; Teixeira et al., 2007; Tezer et al., 2008; Tinuper et al., 2003; Widdess-Walsh et al., 2005). A waiting or latent period suggests that epileptogenic mechanisms are developing but not yet capable of producing epileptiform activity. This then makes the latent period a critical time for identification of epileptogenic mechanisms, particularly since seizures themselves can alter physiological processes (Mathern et al., 2002) and exacerbate hyperexcitability (Isaeva et al., 2010).

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The rodent neonatal freeze lesion model of microgyria replicates the histopathological features of human 4-layered polymicrogyria (Dvorak et al., 1978; Dvorak and Feit, 1977; Jacobs et al., 1996), with a focal four-layered malformed region missing deep layers present by P7 (Rosen et al., 1992). We have previously suggested that the absence of layer IV within the microgyrus triggers reorganization of afferent inputs, particularly thalamocortical ones that find the appropriate laminar targets in the cortex surrounding the malformation, the paramicrogyral region (PMR) (Jacobs et al., 1999b). This was subsequently supported by anatomical studies showing that afferents avoid the microgyrus and accumulate in the PMR (Rosen et al., 2000). The physiological evidence of increased excitatory input to this region further suggested that this remapping of afferents might contribute to the expression of epileptiform activity that occurs with an abrupt onset on P12 (Jacobs et al., 1999a). To confirm that excitatory hyperinnervation could be a cause rather than a result of hyperexcitability, we have previously examined whether it occurred prior to or after the onset of network hyperexcitability at P12. We found that there was an abrupt and consistent increase in the frequency of mEPSCs in PMR pyramidal neurons on P10 (Zsombok and Jacobs, 2007). When neonatally freeze-lesioned animals are given a second hit via hyperthermia on P10, they develop spontaneous seizures as adults (Scantlebury et al., 2005). Since many alterations of microgyral and PMR cortex other than the increased excitatory afferents are known, it is important to determine if it is indeed the presence of this increased afferent input that initiates the epileptogenesis.

To examine this we have asked if there is an increased susceptibility for epileptiform activity during the latent period, particularly coinciding with the hyperinnervation on P10. We have tested this in vitro with a two-hit model of neonatal freeze lesion followed 6 or more days later by the presence of low-Mg²⁺ aCSF. Low-Mg²⁺ aCSF has commonly been used to test for hyperexcitability and models a general overall increase in cortical excitability.

Methods

Freeze Lesion

On postnatal day (P) one, Sprague Dawley rat pups were anesthetized in ice for ~4 minutes. When movement and response to tail pinch ceased, an incision was made through the scalp. With the skull exposed, a freezing (-50° C) rectangular probe (tip size = 2 × 5 mm) was placed over the somatosensory cortex for 5 seconds. The scalp was then sutured and the pup was placed under a heat lamp to warm, and ~10 minutes later returned to the dam. For a detailed description of procedure see (Jacobs et al., 1996).

Brain Extraction and Slice Preparation

Between P7 and P38, rats were anesthetized with pentobarbital (55 mg/kg i.p.) or isoflurane exposure and decapitated for brain removal. Once the brain was removed it was immediately chilled in sucrose-modified artificial cerebral spinal fluid (aCSF) containing: (in mM) 2.5 KCl, 10MgSO₄, 3.4 CaCl₂, 1.25 NaH₂PO₄, 234 sucrose, 11 glucose, and 26 NaHCO₃. Coronal 400 μm thick slices were cut in modified aCSF with a 1000plus vibratome. Once cut, the slices were placed in an oxygenated *normal* aCSF containing: (in mM) 126 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 10 glucose, and 26 NaHCO₃. The slices remained in this solution at 34° C for 30-45 minutes and at room temperature thereafter until placed in the recording chamber.

Electrophysiological Recordings

Slices were placed in an oxygenated interface chamber with 34° C normal aCSF flowing over the slice. Field potential recordings were made using glass micropipettes (2-8 MΩ, 1 M

NaCl), placed within superficial layers (II/III) ~1 mm lateral to the microsulcus in PMR or in homotopic control (unlesioned) cortex. We have previously demonstrated that the PMR is the most sensitive region for epileptiform activity (Jacobs et al., 1999a). For extracellular stimulation, a concentric bipolar electrode was placed at the interface of white and gray matter directly beneath the recording site, such that these two electrodes were in a plane orthogonal to the pia. A single current pulse (20 μ sec long) of varying current intensity (0.30-10 mA, defined as threshold current) was applied to generate a short latency field potential with a peak negativity of 200 μ V. To further assess slice health and to estimate intrinsic cortical excitability, a series of increasing stimuli (stimulus intensity protocol) were applied by successively increasing the duration of the pulse (1 $\times$, 2 $\times$, 4 $\times$, 8 $\times$, and 16 $\times$). Slices were deemed viable if they met two criteria: 1) the short latency response increased in a fashion that was graded with stimulus intensity; and 2) at a stimulus intensity of 16 $\times$ threshold, the field potential negativity had a peak of at least 0.6 mV. Field potentials were amplified 1000 $\times$ (AxoClamp 2B, Axon Instruments and FLA-01 amplifier, Cygnus Technologies) and digitized at 5-10 kHz with a Digidata 1322a (Axon Instruments) and recorded to hard drive with Clampex software (Axon Instruments). In order to test the susceptibility to epileptiform activity, before and after low-Mg²⁺ aCSF, two sec of response to threshold current stimulation was recorded once a minute. After 10 minutes of recordings in normal aCSF, low-Mg²⁺ aCSF (normal aCSF without the MgCl₂ added) was applied for the remainder of the experiment (100 minutes). With our chamber, the solution required five minutes to reach the chamber.

Data Analysis

Measures of the peak, area, and time to peak were made on three sweep averages of the short latency field potential negativity in response to the stimulus intensity protocol using IGOR software (Wavemetrics). Prior to measurement calculation, the responses were zeroed based on the time from zero to nine msec, with the stimulus occurring 10 msec into the sweep. Examination of the peak and area of responses began just after the stimulus artifact. Differences between control and PMR cortex were analyzed using 2-way ANOVAs (intensity versus experimental group). Differences within experimental group between age groups were also tested using 2-way ANOVAs. Epileptiform activity was detected and sorted into interictal and ictal-like (hereafter called ictal) activity by an automated epileptiform detection module written in IGOR by Jeremy Bergsman. Interictal events were detected as peaks occurring after the short latency response that were at least 2 $\times$ the baseline voltage of the Gaussian filtered (70 Hz) raw field potential trace (see Fig. 2D). Ictal events were detected as peaks that were at least 2 $\times$ the baseline of the derivative of the filtered trace (see Fig. 2F, H). In both cases if the threshold of 2 $\times$ the baseline produced artifacts on manual examination, then the threshold was raised to as much as 3 $\times$ the baseline. Sweeps with two or more ictal events were considered repetitive ictal. Each sweep was processed for all three types of epileptiform activity with ictal detection overriding interictal and repetitive ictal overriding single spike ictal. The percentage of epileptiform activity incidence was calculated by counting the number of epileptiform sweeps evoked within 50 sweeps after the initial epileptiform sweep. The area of the rectified late (490 to 1990 msec) activity was measured with Clampfit software (Axon Instruments). Because comparisons between control and PMR cortex were truly independent for each age group, Bonferroni adjusted t-tests were used to detect significant differences ($p < 0.05$) for measures of amount of epileptiform activity. For the measure of time to first epileptiform sweep, age appeared to be a factor for the PMR group, thus we used a 2-way ANOVA to determine if there were differences between PMR and control responses as well as differences among age groups.

Spectrograms were created by importing event driven data collected with Clampfit 8.2 (Axon) as gap free data in Matlab (7.9.0, 2009b) using a custom m-file. The initial 400 μ s of

each sweep was eliminated to reduce the effect of the stimulus artifact on the generated spectrogram. For this study, spectrograms with a 0.5 Hz frequency resolution were plotted from 0.0 to 15 Hz.

Results

In order to understand the onset of excitability changes in malformed cortex, responses from four age groups were characterized: a) P7-9; b) P10-11; c) P12-15; and d) P28-36. The first group, P7-9, reflects a time prior to the increase in excitatory afferent input to layer V pyramidal neurons reported in this malformation model (Zsombok and Jacobs, 2007). The second group, P10-11, reflects an age when these excitatory afferents are increased, yet field potential epileptiform activity cannot be evoked under conditions of normal aCSF. In the third age group, P12-15, epileptiform activity is readily and consistently evoked from the PMR (Jacobs et al., 1999a), although control cortex also shows an increase in excitability at these ages (Luhmann and Prince, 1990; Luhmann and Prince, 1991). In the final, adult age group, the PMR continues to show epileptiform activity while control cortex is at its lowest level as measured by evoked field potentials.

Intrinsic excitability

Although there is no epileptiform activity evoked within the PMR at P10-11 in normal aCSF, we reasoned that the increase in excitatory afferents observed at these ages (Zsombok and Jacobs, 2007) might be reflected within the peak or area of the short latency evoked response in normal aCSF. To test this, an extracellular stimulus was applied to deep layers while recording the network response as field potentials from the overlying superficial layers. The current required to evoke the threshold response was not different between control and PMR for any age group (t-tests, $p > 0.05$, N.S.). A series of increasingly intense stimuli was applied (see Methods) and the area of the short latency field potential negativity examined. As expected, control and PMR short latency responses did not vary for the P7-9 age group (Fig. 1A). Surprisingly, there was also no difference between control and PMR groups at ages P10-11 (Fig. 1B). In contrast, for the P12-15 age group, the PMR cortex had responses more than twice as large for stimulus intensities of 4× and greater (Fig. 1C, 2-way ANOVA, $p < 0.001$). In the adult age group, the PMR responses were also significantly larger than control (Fig. 1D, 2-way ANOVA, $p < 0.05$). The PMR responses at P12-15 were larger than any other responses at any other time. In all cases, the same result was obtained with the measure of peak field negativity (not shown).

Effect of 2nd Hit

We sought to determine whether there was an underlying *susceptibility* to epileptiform activity at the ages corresponding to the increase in excitatory afferents (P10-11). To investigate this, we employed a two-hit model, using low-Mg²⁺ aCSF to increase overall cortical excitability. The PMR and homotopic control cortex were stimulated once a minute at the threshold current. After 10 minutes of normal aCSF, the bathing solution was switched to low-Mg²⁺ aCSF and the responses followed for another 100 min. An automated system was used to detect evoked epileptiform events and to characterize them as interictal or ictal (Fig. 2, also see Methods). Events characterized as interictal were slower, particularly in time to peak, and typically larger in amplitude and duration as compared to ictal events. In addition, ictal events often occurred repeatedly within the same sweep (2 sec post-stimulus period, Fig. 2D).

Low-Mg²⁺ aCSF produced epileptiform activity in all slices from both control and PMR cortex. The changes in field potential activity occurring over the course of an individual experiment were easily visualized in spectrograms displaying power versus frequency over

experiment time (Fig. 3A). While the short latency activity produced high power at low frequencies (0-1.5 Hz), epileptiform activity produced increased power at frequencies above 1 Hz. Over the course of an experiment, the epileptiform activity typically evolved from slower, interictal events to fast and ultimately repetitive ictal activity (Fig. 3B). This pattern was similar in both control and PMR cortex, but typically occurred earlier for PMR than controls.

Effect of Age on Epileptiform Activity Incidence

In order to examine changes in susceptibility for epileptiform activity, three aspects were analyzed: incidence of events (interictal and ictal), time to first epileptiform event, and magnitude of the late field potential activity. Confirming previous descriptions of increased excitability in the second to third postnatal week in rodents (Jensen et al., 1991; Luhmann and Prince, 1990), our results show that for control cortex, the P12-15 age group was the most excitable, with significantly more total epileptiform sweeps (Fig. 4A-D, I, 1-way ANOVA, $p < 0.005$, LSD posthoc) and significantly more single spike ictal than other age groups (Fig. 4E-H, 1-way ANOVA, $p < 0.05$, LSD posthoc). The adult age group in controls had the fewest sweeps with epileptiform activity, including no ictal activity (Fig. 4H). The PMR cortex showed similar trends with age, however there was not a significant effect of age with either total epileptiform activity or ictal incidence (1-way ANOVAs, N.S.). Both control and PMR also showed a significant positive correlation between incidence of ictal activity and age for ages up to P15 ($R = 0.62$ for control, and 0.39 for PMR).

Incidence of epileptiform events in PMR versus Control

In PMR compared to control cortex, epileptiform activity not only occurred earlier within individual experiments, but also more often and with greater severity than in control cortex from the same age groups (Figs. 3-5). For all age groups, there was an increased incidence of epileptiform activity in PMR relative to control cortex (Fig. 4). In the P7-9 age group, the majority of the epileptiform events were in the form of interictal activity for both control and PMR cortex (Fig. 4E). In this age group, the increase in epileptiform activity within PMR cortex was due specifically to an increase in interictal events (Fig. 4E). In the P10-11 age group, the contribution of ictal sweeps to the total number of epileptiform sweeps was significantly larger relative to that for the P7-9 age group in control cortex: 28 ± 8 versus $55 \pm 13\%$ of epileptiform sweeps showed ictal activity for 11 and 8 control slices in the P7-9 and P10-11 age groups, respectively (t-test, $p < 0.05$). The same was true for PMR cortex: 21 ± 8 versus $57 \pm 10\%$ of epileptiform sweeps showed ictal activity for 12 and 10 slices in the P7-9 and P10-11 age groups, respectively (t-test, $p < 0.005$). Comparing PMR to control within the P10-11 age group, PMR cortex had significantly more epileptiform activity due to a greater percentage of interictal sweeps (Fig. 4B, F). In the P12-15 age group one of seven (14%) control slices had interictal epileptiform prior to application of low- Mg^{2+} aCSF, while this was true for three of seven (43%) PMR slices. In no case was ictal activity ever observed prior to application of low- Mg^{2+} aCSF. Similar to the younger age groups, for P12-15, the increase in percentage of epileptiform sweeps for PMR compared to control cortex was due to an increase in interictal activity (25 ± 0 versus $52 \pm 0\%$ of sweeps contained interictal activity for control and PMR respectively, $P = 0.05$, t-test, Fig. 4C,G). Even when the sweeps prior to application of low- Mg^{2+} aCSF were excluded, the PMR still had a significantly larger percent of epileptiform sweeps compared to control (65 ± 0.05 versus $82 \pm 0.03\%$ epileptiform sweeps for 7 control and 7 PMR slices, respectively, t-test, $p < 0.05$). In this age group, the proportion of epileptiform sweeps that contained ictal activity was similar for control and PMR cortex (59 ± 8 and $42 \pm 12\%$ of epileptiform sweeps for control and PMR, respectively). In the adult age group, none of the control, but all of the PMR slices exhibited ictal activity (Fig. 4H). Thus, only in this age group was the increase in epileptiform sweeps for PMR compared to control cortex due to ictal and not interictal

activity. The proportion of PMR ictal sweeps for this age group was similar to that for the previous two age groups ($52 \pm 20\%$ of epileptiform sweeps).

Threshold for epileptiform activity

The time to the first epileptiform activity after application of low-Mg²⁺ aCSF is another measure of susceptibility to hyperexcitability, but more specifically measures the threshold for epileptiform events. In PMR slices, the mean time to first epileptiform sweep was less than 10 minutes after low-Mg²⁺ aCSF application, for all age groups (Fig. 5). The results of the 2-way ANOVA show that the threshold for epileptiform activity is less in the PMR than in control cortex ($p < 0.05$). There was no effect of age nor was there a significant interaction between age and experimental condition.

Magnitude of late activity

The magnitude of field potential activity, particularly at the site of the largest current sink (typically in deep layer III, Aizenman et al., 1996), is a reflection of the size of the population EPSC and indirectly the summing IPSCs (Mitzdorf, 1985), as well as the number of cells participating when the presynaptic neurons are within the same structure. This is the case for both the short latency event as well as longer latency epileptiform activity. To determine if malformed cortex had a larger network participating in the generation of epileptiform events or enhanced summation of PSCs in PMR cortex, we analyzed the rectified area of the field potential response occurring after the short latency event ended (500 – 2000 msec, late rectified field potential, LRFP, Fig. 6A). Rectification was necessary since epileptiform events often cross the baseline. Each age group showed a distinct pattern in this measure over the course of the experiment (Fig. 6C-F). For the P7-9 age group, the PMR cortex showed a significant increase relative to control cortex in the LRFP (Fig. 6C, 2-way ANOVA, $P < 0.001$). This increase was most pronounced just after application of the low-Mg²⁺ aCSF (Fig. 6G, adjusted Bonferroni t-test, $P < 0.01$). The responses from both control and PMR however reached a similar maximum by 50 min after the solution change (Fig. 6C, G). For the P10-11 age group, the LRFP was increased in the PMR (Fig. 6D, 2-way ANOVA, $P < 0.05$). This age group was the only one that showed an increase in LRFP *prior* to application of the low-Mg²⁺ aCSF for PMR relative to control cortex (Fig. 6H, adjusted Bonferroni t-test, $P < 0.01$). This increase in LRFP for PMR cortex persisted for the duration of the experiment (Fig. 6D, H). In the P12-15 group, there was no difference between PMR and control cortex until ~60 min after onset of low-Mg²⁺ aCSF (Fig. 6E, 2-way ANOVA, $p < 0.05$). Ultimately, control cortex obtained a larger maximum LRFP than did the PMR (Fig. 6I, adjusted Bonferroni t-test, $p < 0.01$). In the adult age group, the control cortex failed to show an increase in LRFP after low-Mg²⁺ aCSF application. (Fig. 6F, J). In PMR cortex the LRFP increased at a slower rate than at younger ages, but reached a level near to that obtained for PMR in the P12-15 age group (Fig. 6I, J), and was significantly greater than control (Fig 6F, 2-way ANOVA, $p < 0.05$), specifically during the last two time points, (Fig. 6J, adjusted Bonferroni t-test, $p < 0.01$).

Discussion

Here we have demonstrated an early susceptibility for increased levels of hyperexcitability in the freeze lesion model of microgyria. Surprisingly, the age of onset for increased susceptibility does not match the timing of one of the previously identified potential epileptogenic mechanisms, that of increased excitatory afferents to layer V pyramidal neurons (Zsombok and Jacobs, 2007). We have also suggested that alterations occur in specific interneuron subtypes (George and Jacobs, 2006; George and Jacobs, 2011), and some effects on interneurons suggest anti-epileptogenic effects (Brill and Huguenard, 2010; Jacobs and Prince, 2005). Yet to be determined is the onset timing for these pro and anti-

epileptogenic interneuron effects. Early susceptibility followed by delayed onset of epileptiform activity in normal aCSF also suggests that network expression may require maturation of particular cellular properties in the presence of previously occurring abnormalities.

Developmental Changes in Excitability

A number of previous findings in malformation models suggested that early insults may 'freeze' the cortex in an immature state or at least delay maturation. In rodent and cat models of microgyria, these included a maintenance of radial glia into adulthood (Rosen et al., 1994), increased duration of the action potential (Luhmann et al., 1998), a maintenance of normally pruned connections from auditory to visual cortex (Innocenti and Berbel, 1991) and enhanced functional response to an immature form of both the GABA_A and the NMDA receptors (Bandyopadhyay and Hablitz, 2006; Defazio and Hablitz, 2000; Hablitz and Defazio, 2000). Further, there is evidence that a delay in the normal developmental decrease of intracellular Cl⁻ concentration (caused by a delayed transition in cation-Cl⁻ transporters from NKCC1 to KCC2 expression) promotes an immature state in which GABAergic transmission remains excitatory for a longer time (Shimizu-Okabe et al., 2007; Wang et al., 2012). These effects largely occurred within the malformed region rather than the adjacent area of hyperexcitability. Our current results suggest that hyperexcitability does not arise as a result of a simple maintenance of an earlier hyperexcitable state. This is true since the most excitable age for controls was P12-15, yet the increased level of epileptiform activity was already present prior to this age. The increase in ictal events does not occur until the adult age group, suggesting that there is a continued development of epileptogenic processes, rather than a static mechanistic abnormality, but also that a chronic state of hyperexcitability is ultimately induced in this model. Previous studies have suggested that in fact ictal and interictal mechanisms may develop independently (Avoli, 2001; Avoli and Barbarosie, 1999; De Curtis and Avanzini, 2001). Our data suggest further that independent latent periods exist for expression of interictal activity without a second hit, and susceptibility to ictal activity. Although for some models the malformation is induced in utero, expression of hyperexcitability is delayed to weeks postnatally (Cobos et al., 2005; Trotter et al., 2006; Zeng et al., 2011). Changes in susceptibility over development have not been well-studied in malformation models and as shown here, could occur substantially earlier than when seizures or in vitro hyperexcitability is present as has been shown recently for a non-malformation model (Rattka et al., 2011).

Latent Period

Recent studies have suggested that development of convulsive seizures occurs gradually, rather than suddenly after a latent period (Bragin et al., 2009; Dudek and Staley, 2011). For at least some epilepsy models, non-convulsive seizures precede convulsive ones, suggesting an increasing severity (Williams et al., 2009). Here we find that susceptibility for increased interictal activity is present long before the expression of such activity under conditions of normal aCSF (at P12). This could result from a progressive nature of the interictal activity, although our data do not support this idea. The measures of percentage of epileptiform activity, time to first epileptiform sweep and even magnitude of late activity do not get progressively worse from P7 to P12. Although the P12-15 group was the most hyperexcitable in PMR as well as control cortex, the difference between the excitability for PMR relative to control was actually lowest for this age group (see Fig. 4I). A second explanation for the delayed expression in the presence of susceptibility is that a normal maturational process may be necessary for this expression. Previous explanations for the normal increased excitability seen at 2-3 weeks postnatally in control rats include changes in both NMDA and GABA receptor development (Luhmann and Prince, 1990; Luhmann and Prince, 1991; Velisek and Moshe, 2008). In adult freeze-lesioned rats, NMDA receptor

binding is increased while that of GABA_A is decreased over control levels (Zilles et al., 1998). These effects are most prominent within the malformed region, but also present in the surround. The onset timing of these effects are unknown, however if they alone created the hyperexcitability, the malformed region would be expected to be the most excitable region in this model, however it is less excitable than the PMR (Jacobs et al., 1999a). Thus changes in NMDA and/or GABA_A receptors are unlikely to create the hyperexcitability, however they may influence the expression of it based on developmental changes, including increased expression of NMDA in superficial layers.

Although we used an activation of NMDA receptors as the second hit here, we expect that other means of increasing excitability would produce the same effect. Blocking GABA_A receptors is a common method to increase excitability in cortical slices and would be expected to be very effective in this malformed tissue, particularly if done when GABA_A receptors are already reduced (Zilles et al., 1998). In fact this early susceptibility that does not coincide with increased excitatory drive to pyramidal neurons may instead be due to changes in inhibitory neurons or in connectivity to them (George and Jacobs, 2006; George and Jacobs, 2011), that is more subtle and therefore only expressed with the increased excitability.

The decrease in excitability in mature animals compared to the 2nd-3rd postnatal week has also been proposed to be due to an increased postictal refractoriness based on kindling experiments (Velisek and Moshe, 2008). The difference in ictal activity that we found between control and PMR may reflect a lack of development of this refractoriness or the increased NMDA receptors responding to the lack of Mg²⁺ block.

Conclusions

We have found an early susceptibility for interictal activity in malformed brain, as well as a chronic susceptibility for ictal activity. Once established, there appears to be a maintained rather than progressive effect of the susceptibility for interictal activity. We conclude from this that the delay between this susceptibility and the expression of increased interictal activity without the second hit may be due to normal maturation of characteristics that increase excitability even in control brains combined with the abnormalities such as the previously demonstrated increased excitatory afferents. The separate time course for susceptibility to increased levels of ictal activity suggests that this may arise from separate mechanisms. This study highlights the importance of determining the timing of susceptibility for epileptiform activity in order to identify the underlying mechanisms.

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After induction delay to onset of epileptiform activity occurs in malformed cortex.

We hypothesize that increased susceptibility to epileptiform activity occurs during this delay.

A 'second hit' of low magnesium aCSF was used to test this hypothesis.

Increased incidence of epileptiform activity occurs by postnatal day 7 in rat.

Epileptic mechanisms are present earlier than previously thought for malformations.

Figure 1.

Intrinsic excitability changes in normal aCSF during development: Stimulus intensity series profiles for immature (A: P7-9; B: P10-11; C: P12-15) and adult (D: P28-36) age groups, for control (gray) and PMR (black) cortex. Typical responses from each experimental group are shown in inset, for control (left), and PMR (right), with stimulus artifact masked. For control and PMR respectively, $n = 15$ and 17 (P7-9); 13 and 10 (P10-11); 28 and 40 (P12-15); 6 and 7 (P28-36) slices. * = significant difference, 2-way ANOVA, $p < 0.05$.

Figure 2.

Example of automated epileptiform activity detection. Left: Raw sweep examples of: short latency activity only (A); interictal (C); single spike ictal (E); and repetitive spike ictal activity (G). Right: Preparation of trace on left for detection of a particular type of epileptiform activity. Traces were filtered at 70 Hz to detect interictal activity (B, D). The derivative of the filtered trace was used to detect ictal events (F, H). Dashed lines above traces depict detection threshold, which was set to 2-3 times baseline of the prepared wave. Solid lines above traces indicate location of detected peak. Scale bars for A-D found in B, E,G found in E, F,H found in F.

Figure 3.

After application of low-Mg²⁺ aCSF at time 0, epileptiform activity occurred earlier and more frequently in PMR cortex compared to controls. (A) Spectrograms of field potential responses to threshold stimulus given to deep layers below recording. Activity occurring in between stimulus presentations (58 sec) is not shown. The x-axis plots the time over the course of individual experiments for control (top panel) and PMR (bottom panel). 'Hot' colors indicate higher power field potential activity (mV/ms²). Black arrows point to the first epileptiform sweep. Blue arrows at P12-15 point to the example traces shown in B (sweeps 5, 20, 66, and 98). (B) Examples of the progression of epileptiform activity through an individual experiment in control (top) and PMR (bottom) cortex. Epileptiform activity begins as slow, large amplitude events and evolves into repetitive sharp 'spikes'.

Figure 4.

Increased Incidence of Epileptiform Sweeps in PMR Cortex. (A-D) Percentage of sweeps with evoked epileptiform activity for age groups (control, PMR slices): P7-9 (A, 11, 12 slices); P10-11 (B, 8, 10 slices); P12-15 (C 7, 7 slices); and P28-40 (D 4, 4 slices). * = significant difference between control and PMR (t-tests, $p < 0.05$). (E-H) Distribution of the type of epileptiform activity observed for each group: Interictal, light gray; Single spike (SS) ictal, dark gray; and Repetitive spike (RS) ictal, black. The following symbols represent significant differences between control and PMR: # = interictal, % = SS ictal, and * = RS ictal (t-tests, $p < 0.05$). (I) Percentage of evoked epileptiform activity relative to the average amount observed in control P28-36 (100%) for control groups (gray filled symbols with black outlines) and PMR groups (black filled symbols with gray outlines), for P7-9 (diamonds), P10-11 (triangles), P12-15 (circles), and P28-36 (squares).

Figure 5. Time to first epileptiform sweep after low- Mg^{2+} Application. Control on left, PMR age groups on right. N = 12, 10, 7, and 4 control slices and 11, 8, 7, and 4 PMR slices for age groups P7-9, P10-11, P12-15, and P28-36, respectively. * = $p < 0.05$, 2-way ANOVA.

Figure 6.

Magnitude of late field potential activity. (A) Typical raw field potential sweep. (B) Same sweep as in A, with late activity (490 – 1990 msec) rectified. (C-F) Plots of the area of the rectified late field potential activity before (0-9.99 min) and after (10-100 min) low-Mg²⁺ application, for control (gray circles) and PMR (black squares). Age group indicated at top of plot. Activity was averaged over 5 min (5 stimulus presentations) before averaging across subjects within group. Error bars indicate SEM. Vertical dashed line indicates when low-Mg²⁺ aCSF reached chamber. (G-J) Bar graphs comparing control (gray) to PMR (black) for data shown above, but averaged within three time periods: Early (sweeps 1-15), before low-Mg²⁺ reaches the slice; Middle (sweeps 30-50), after effect of low-Mg²⁺ is apparent; and Late (sweeps 50-110) when maximum effect has stabilized. * = $p < 0.05$, 2-way ANOVA.